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14. ABSTRACT

Due to the PI's move from Brigham and Women's Hospital to Cornell University on July 1, 2011, no research was conducted at Brigham and Women's Hospital during the report period (July 1 – Dec 31, 2011). Nonetheless, while waiting for the award transfer to be completed (the official project period start date at Cornell was Feb 1, 2012), we already accomplished several goals of the proposed statement of work.

We created panels of cell lines based on two model systems (MCF10A – normal epithelial cells; MDA-MB-231 metastatic breast cancer cells) with systematic alterations in the expression of lamins A, B1, B2, C, and lamin B receptor (LBR). We then evaluated the effect of altered lamin expression on nuclear stiffness in these cell lines. While increased expression of lamin A caused stiffer, less deformable nuclei, reduction of lamins A/C expression by shRNA reduced nuclear stiffness. The effect of alterations in other lamins was less substantial. However, expression of LBR resulted in increased lobulation of the nucleus. These preliminary data support our hypothesis that changes in expression of nuclear envelope proteins recently reported in breast cancer is sufficient to cause altered nuclear morphology and stiffness, which could result in altered migration and invasion.

15. SUBJECT TERMS
Lamins, MCF10A, MDA-MB-231, nuclear stiffness, nuclear shape, lamin B receptor

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INTRODUCTION

The central hypothesis of this proposal is that changes in the expression of nuclear envelope proteins such as lamins or lamin B receptor (LBR) may contribute to the characteristic irregular morphology of cancer cell nuclei and directly modulate cellular functions relevant to cancer progression. Nuclear lamins, particularly lamins A and C, are important determinants of nuclear shape and stiffness.[1-3] At the same time, these proteins also interact with various transcription factors, thereby affecting important signaling pathways.[1, 4] The purpose of this study is to conduct a systematic analysis of the functional consequences of changes in the expression of lamins (A, B1, B2, and C) and lamin B receptor on nuclear morphology and stiffness, as well as the functional consequences of such changes on cell migration through confined spaces (where more deformable nuclei may facilitate enhanced passage), proliferation, and epithelial-to-mesenchymal transition (EMT). In addition, we proposed to conduct an analysis of samples derived from breast cancer patients and orthotopic mouse models of the disease to assess changes in the expression of nuclear envelope proteins in breast cancer samples.

BODY

Due to the move of the PI from Brigham and Women's Hospital to Cornell University in 2011, there had been some delays in the transfer of this award to the new institution, as well as the time setting up the laboratory at Cornell, prompting us to request a no-cost extension to 7/31/2014 that was granted on 3/7/2013. Despite these circumstances, we have made significant progress in all of the tasks outlined in the updated SOW.

***Task 1:* Acquire a panel of cell lines and patient-derived samples representing various stages of breast cancer progression from benign to metastatic (Months 0–18)**

As outlined in the previous progress report, we have focused our initial efforts on a subset of cell lines to optimize experimental techniques. We have successfully modified the following cell lines to either overexpress lamins (A, B1, B2, C) and LBR or to stably knockdown the expression of these nuclear envelope proteins: MCF10A (normal mammary epithelial cells), MDA-MB-231 (metastatic breast cancer cells), and MCF7 (non-metastatic breast cancer cells). The characterization of these cells was already described in detail in the previous progress report. In this progress report, we focus on the functional results obtained with the MDA-MB-231 cell line, as we believe that it represents the most relevant model for aggressive breast cancer. Nonetheless, as I recently joined the Cornell University Center on the Microenvironment and Metastasis that is part of the National Cancer Institute Physical Science in Oncology (PSOC) initiative, we are currently collaborating with other laboratories at Cornell University to obtain additional breast cancer cell lines to validate our results.

To obtain patient samples, we established a collaboration with Linda Vahdat at Weill Cornell Medical College, who has already provided us with a set of paraffin embedded tissue sections that we used to assess expression levels of nuclear envelope proteins by immunofluorescence and immunohistochemistry. Results from the first set of samples are presented under the section for Task 4.

Regarding the collection of tissue sections from a breast cancer mouse model, we are working with Claudia Fischbach's laboratory at Cornell University, who has already provided us with samples to optimize our staining procedures.

<u>Task 2:</u> Modulate nuclear shape and stiffness in a panel of well characterized breast cancer cells and non-tumorigenic controls by stable, ectopic expression of lamins A, B1, B2, C, a dominant negative lamin A mutant, or lamin B receptor (LBR) (Months 1–18)

We created a panel of cell lines derived from MCF10A and MDA-MB-231 cells in which we selectively overexpressed lamin A, lamin B1, lamin B2, lamin C, or LBR with a custom-designed retroviral construct followed by fluorescence activated cell sorting to obtain physiological expression levels. In addition, we created a corresponding panel of cell lines in which we reduced expression of lamins A/C, lamin B1, lamin B2, and LBR by shRNA mediated knockdown. Changes in protein levels were confirmed by Western blot analysis as described in the previous progress report. As we experienced that even in these stably modified cells lamin expression can revert back over time, presumably due to strong selection pressure, we have also created clonal populations of the modified cells, which should be more resistant to population drift. In addition, we are considering repeating some of the functional experiments with transient siRNA mediated knockdown to confirm results obtained with the stably modified cells.

To assess the effect of altered nuclear envelope protein expression on nuclear deformability, we had previously subjected the panel of modified MCF10A cells to substrate strain experiments and measured the resulting nuclear deformations (see previous progress report). These experiments revealed that increased expression of lamin A significantly increased nuclear stiffness, while reduced expression of lamins A/C dramatically decreased nuclear stiffness. The effects of changes in the expression of other nuclear envelope proteins were less dramatic, but still significant, particularly for overexpression of lamin C, lamin B1, and LBR, which reduced nuclear stiffness. One limitation of the substrate strain experiments we discovered was that several cancer cells, including the MDA-MB-231 and particularly the MCF7 cells, only weakly adhere to the substrate and tend to detach when subjected to strain, making the interpretation of the strain analysis challenging. To circumvent this problem and enable the analysis of nuclear mechanics in breast cancer cells independent of cell adhesion properties, we have pursued two alternative avenues. In the first approach, we are collaborating with the group of Peter Friedl and Katarina Wolf at the University of Nijmegen to measure nuclear deformability using atomic force microscopy (AFM). These experiments have already yielded encouraging results. For the second approach, we have developed a novel experimental technique in which suspended cells are perfused through a microfluidic device with microscopic constrictions smaller than the size of the nucleus while imaging the transit of the cell through the constrictions with a high speed camera on a microscope (Fig. 1).[5]

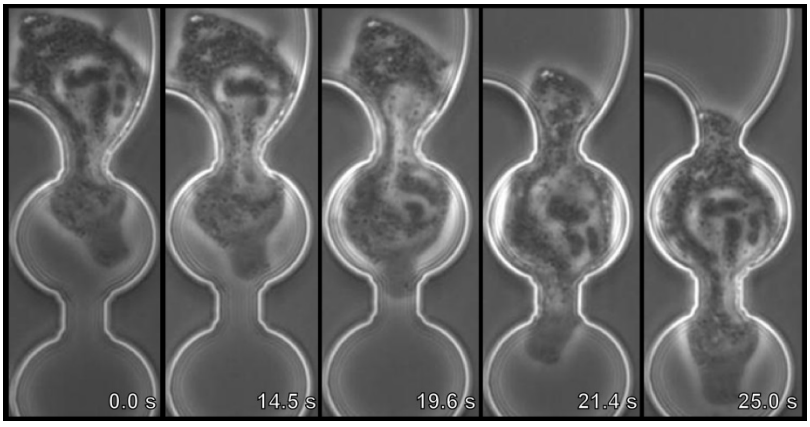

Figure 1. Perfusion of MDA-MB-231 cells through microfluidic constriction channel

We validated our device with lamin A/C-deficient (*Lmna*$^{-/-}$) and wild-type mouse embryo fibroblasts, which we had previously characterized by substrate strain analysis.[3] These experiments showed that lamin A/C-deficient cells passed significantly faster through 5µm constrictions than cells from wild-type littermates (data not shown). Our preliminary experiments on MDA-MB-231 cells revealed that cells with reduced expression of lamins A/C were able to pass through the 5µm constrictions significantly faster than comparably sized cells with normal levels of lamins A and C (mock controls). As cell size varied between the various populations and it was recently shown that the transit time of cells through narrow constriction exponentially increases with cell size,[6] we also plotted the regression curves for MDA-MB-231 cells with reduced levels of lamin A/C and mock controls (Fig. 2). These plots reveal a substantial downward shift of the regression line for the MDA-MB-231 cells, indicating faster transit times for a given cell size.

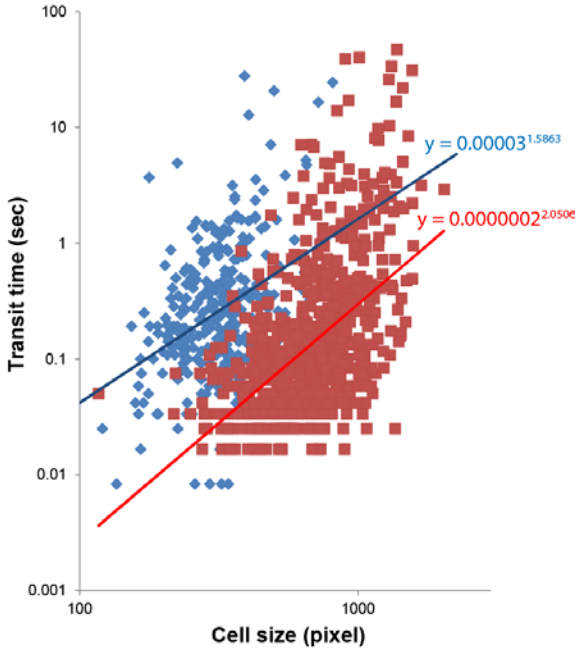

Figure 2. Transit times during perfusion of MDA-MB-231 cells with stably reduced levels of lamin A/C (red) and mock controls (blue) through 5µm constrictions plotted as a function of cell size (log-log plot). Red and blue lines represent the least-squares regression fit for each of the cell populations, revealing that lamin A/C-deficient cells are more deformable than wild-type cells.

<u>Task 3:</u> Investigate whether changes in nuclear shape or stiffness can alter invasion, migration, or perfusion through narrow channels in the newly created panel of cell lines (Months 6–24)

We have already succeeded in designing and generating microfluidic devices to measure the ability of the modified cells to pass through narrow constrictions. Results of the perfusion assay, which we are currently applying to measure nuclear deformability, are depicted above (Fig. 2). To study the effect of altered lamin expression and changes in nuclear deformability on the ability of cells to migrate through narrow constrictions, we fabricated microfluidic devices with precisely engineered constrictions ranging from 2 to 15 µm in size and induced cells to migrate along a chemotactic gradient (EGF) across the channels. We monitored passage of cells through these constrictions at high spatial and temporal resolution and measured transit time, migration speed, and transmigration probability (Fig. 3).

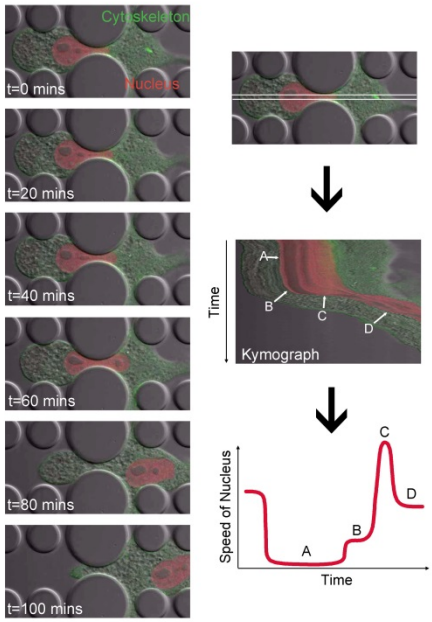

Figure 3. Representative example of a cell with fluorescently labeled cytoplasm (green) and nucleus (red) migrating across a 3 µm constriction along a chemotactic gradient. (Left) Series of frames from time-lapse microscopy acquired over a range of 100 minutes. (Right) Detailed analysis of nuclear movement using kymographs, i.e., plotting the fluorescence intensity profile along a fixed line (thin white rectangle) over time. The resulting kymograph (right, center) shows how the nucleus initially stalls at the constriction (A), then slowly advances (B) before suddenly slipping through the constrictions once it has sufficiently advanced (C). Once the nucleus has passed the constriction, the cell and nucleus resume their normal migration (D).

Our preliminary results suggest that lamin A/C-deficient cells can pass through the constrictions faster than mock-modified cells or cells overexpressing lamin A. We are currently in the process of analyzing a large number of time-lapse sequences to confirm these results and to obtain quantitative parameters of the effect of altered lamin expression on the migration efficiency in these devices. We are encouraged by the recent report of Peter Friedl's group that convincingly demonstrated that nuclear deformability can present a rate-limiting step in the non-protelytic migration of cells through confined spaces.[7] We have already sent some of our modified breast cancer cells to Katarina Wolf and Peter Friedl's group at the University of Nijmegen, so that they can assess the ability of our cells to migrate through dense collagen matrices in their laboratory.

While using our experimental system, we also noticed that the fluorescent label we are using to the mark the nuclei, i.e., GFP or mCherry with a nuclear localization sequence (NLS), can also yield important information on nuclear rupture in cancer cells, which was recently described by the Hetzer group.[8] In our assay, GFP- or mCherry-NLS constructs are normally localized to the nucleus, but will (transiently) leak into the cytoplasm during nuclear envelope rupture and will eventually accumulate back in the nucleus once the nuclear envelope has been resealed. During our time-lapse imaging of cancer cells migrating through the narrow constrictions, we observed that lamin A/C-deficient cells were significantly more prone to (transient) nuclear envelope rupture than mock-modified cells, suggesting that the lamin A/C-deficient cells are more fragile, as we previously demonstrated for lamin A/C-deficient and mutant fibroblasts.[3, 9]

Our microfluidic migration devices also enabled another interesting discovery that we are currently confirming and analyzing in more detail. We observed that cells altered the expression

of lamin A/C during and after passage through the narrow constrictions. In the constriction channels, the expression of lamins appeared to decrease, while lamin A/C expression was significantly increased after successful transit through the constrictions (Fig. 4). In contrast, when cells migrated through larger constrictions that did not require nuclear deformation, we did not observe any changes in lamin expression.

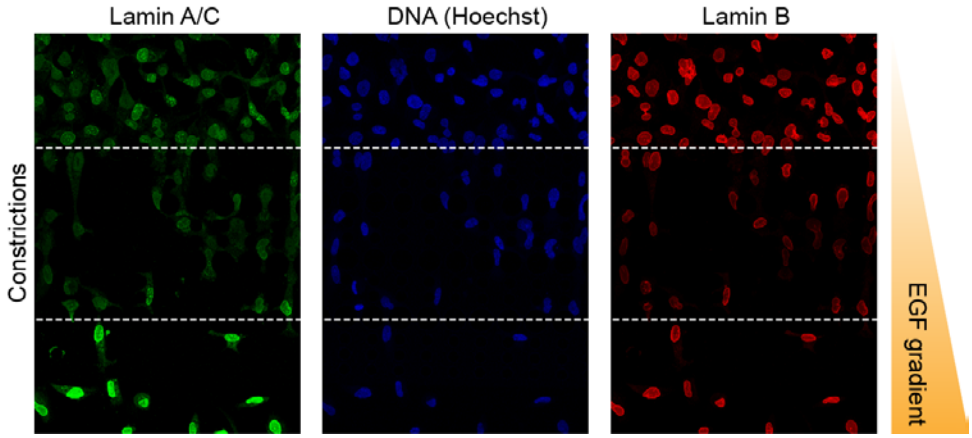

Figure 4. Change in levels of lamin A/C during and after transit through narrow constrictions. MDA-MB-231 cells migrating along a chemotactic gradient (downwards) through microfluidic constriction channels were fixed and immunofluorescently labeled for lamins A/C and lamin B, along with a Hoechst nuclear counterstain. The representative images here suggest that expression of lamin A/C dramatically increases after passage through the area with constrictions (dotted lines).

<u>Task 4:</u> Quantify expression levels of lamins and LBR in breast cancer cell lines, patient-derived breast cancer cells/tissue sections and determine correlation with disease progression from benign to more aggressive/metastatic phenotypes (Months 1–24)

We have already analyzed expression of lamins and LBR in MCF10A, MDA-MB-231, and MCF7 cells. We have also begun with the immunohistochemical analysis of lamin expression in tissue sections provided by Linda Vahdat (Weill Cornell Medical College, New York, NY). Our lamin A/C-staining is working well now (although we are still conducting additional optimization procedures, including with a new antibody from Abcam). We observed highly heterogeneous staining for lamin A/C in many of the cancer tissues, as well as in MDA-MB-231 cells, similar to recently reported findings.[10]

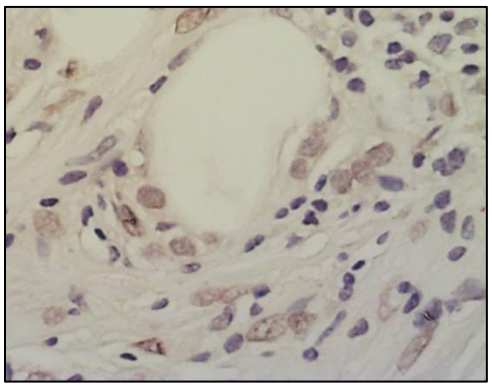

Figure 5. Human breast cancer tissue section stained with H&E (blue) and anti-lamin A/C (brown) revealing heterogeneous staining of cancer cell nuclei for lamin A/C, including some cells completely lacking lamin A/C expression (nuclei appearing blue).

Interestingly, one sample from a triple negative breast cancer showed very high levels of lamin A/C (Fig. 6). According to the oncologist, this patent had had one of the most aggressive breast cancers she had ever seen. We are currently requesting additional patient samples to see whether we can uncover a correlation between lamin expression levels and disease progression/aggressiveness.

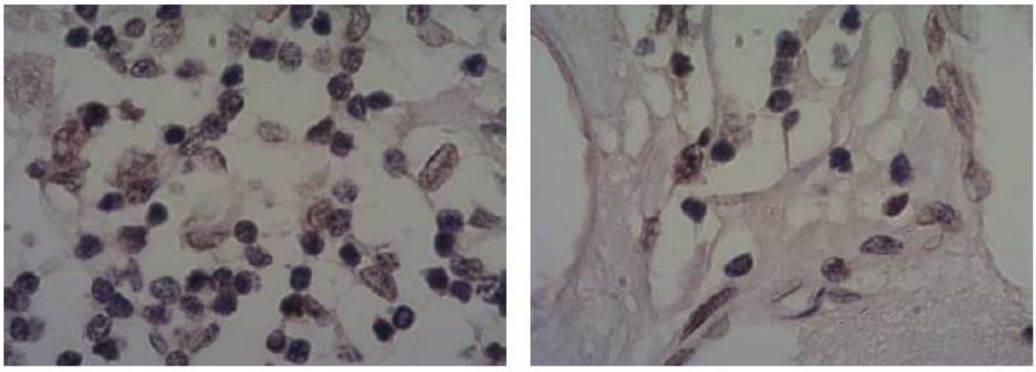

Figure 6. High (but heterogeneous) expression of lamin A/C in a patient with particularly aggressive triple negative breast cancer.

Our hypothesis of altered lamin expression in breast cancer progression is further supported by two recently published reports. In one study, published in the *Chinese Journal of Cancer*, it was described that lamins A/C are absent in almost 40% of human breast cancer tissues and that even in lamin A/C-positive cancers, expression of lamin A/C is heterogeneous or marked by altered intracellular distribution in the tumor cells.[10] Another recent report found that breast cancer tissue has increased expression of lamin B1 compared to normal tissue.[11] Taken together, these findings suggest that altered expression of lamins is prevalent in human breast cancers. Further analysis with a larger panel of samples will help to elucidate whether altered lamin expression can serve as a useful diagnostic and prognostic marker.

Task 5: **Investigate whether changes in the expression of lamins or LBR could modulate biophysical cell functions such as invasion, migration, and perfusion through narrow channels (Months 6–24)**

The outcomes of this task are described under Task 3, as we used modulation of expression of lamins to modify nuclear stiffness. Our preliminary data suggest that reduced expression of lamin A/C enhances the ability of cancer cells to transit through narrow constrictions, but the results are awaiting further confirmation as the quantitative analysis of large number of time-lapse video sequences, each containing dozens of cells, is consuming considerable amounts of time.

Task 6: **Investigate whether changes in the expression of lamins or LBR could modulate non-biophysical cell functions such as proliferation or epithelial-to-mesenchymal transition in the newly created panel of cell lines *(Months 12–24)***

We recently identified a remarkable and surprising effect of loss of lamin A/C on the intracellular localization and activity of the transcriptional coactivator megakaryoblastic leukaemia-1 (MKL1).[12] We found that while in normal cells, MKL1 translocates from the cytoplasm to the nucleus in response to serum stimulation, in lamin A/C-deficient and lamin A/C-depleted cells, MKL1 mostly remains in the cytoplasm. This defect was caused by altered actin dynamics in the

lamin A/C-deficient cells, as nuclear import and export of MKL1 are dependent on its interaction with monomeric G-actin. The altered actin dynamics were a result of the actin-polymerizing function of emerin, which is lost from the nuclear envelope in lamin A/C-deficient cells. As a consequence of disturbed actin dynamics and impaired nuclear translocation of MKL1, lamin A/C-deficient cells had reduced expression of MKL1/SRF target genes, which include actin, actin-binding proteins, vinculin, and serum response factor (SRF). These results were recently published in *Nature*.[12] While some of our findings were originally obtained in the context of cardiac disease caused by lamin A/C mutations, they are also highly relevant to cancer progression, as recent publication suggest a strong link between MKL1 activity and metastatic processes, including epithelial-to-mesenchymal transition (EMT) and cell invasion and migration,[13] including in MDA-MB-231 cells.[14] We have already begun to follow up on these findings to examine MKL1 signaling in MDA-MB-231 cells with altered expression of lamins A/C and to evaluate the effect of altered lamin expression on EMT.

KEY RESEARCH ACCOMPLISHMENTS

- Creation of cell panel based on MCF10A and MDA-MB-231 cell lines with systematic variation in the expression of the nuclear envelope proteins lamin A, lamin B1, lamin B2, lamin C, and lamin B receptor (LBR) by shRNA mediated knockdown or ectopic expression
- Characterization of the effect of changes in nuclear envelope composition on nuclear deformability – increased expression of lamin A caused the most severe increase in nuclear stiffness; decreased expression of lamins A/C resulted in the most severe decrease in nuclear stiffness
- Developed novel microfluidic approach to study nuclear deformability during perfusion of cells through precisely defined constriction channels, promising higher-throughput measurements of nuclear mechanics than traditional methods such as micropipette aspiration, substrate strain application, or atomic force microscopy.
- Developed novel microfluidic devices that enable visualizing cancer cells migrating through precisely defined constrictions at high spatial and temporal resolution, representing a significant advance over traditional Boyden chambers or transwell migration assays.
- Demonstrated that reduction of lamins A/C results in faster transit of cells through narrow constrictions, which might have important implications during perfusion of cancer cells through the circulatory system or proteolysis-independent migration through interstitial spaces.
- Observed increased rates of (transient) nuclear rupture in cancer cells with reduced levels of lamins A/C migrating through narrow constrictions.
- Confirmed that breast cancer tissue samples have altered (and highly heterogeneous) expression of lamins A/C.
- Identified a novel connection between the nuclear envelope proteins lamin A/C and emerin and the transcriptional coactivator MKL1, which fails to properly localize to the nucleus in lamin A/C-deficient cells, resulting in reduced MKL1/SRF activity. As MKL1 is involved in several metastatic processes, these findings could have important implications in cancer progression.

REPORTABLE OUTCOMES

Manuscripts

We published the following manuscripts acknowledging funding from this project.

1. Ho CY, **Lammerding J**. (2012). Lamins at a Glance. *J Cell Sci*. 125 (9): 2087-2093.
2. Isermann P, Davidson PM, Sliz JD, Lammerding J. (2012). Assays to measure nuclear mechanics in interphase cells. *Curr Protoc Cell Biol*. 56:22.16.1- 22.16.21
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4. Zwerger M, Jaalouk DE, Lombardi ML, Isermann P, Mauermann M, Dialynas G, Herrmann H, Wallrath LL, **Lammerding J**. (2013). Myopathic lamin mutations impair nuclear stability in cells and tissue and disrupt nucleo-cytoskeletal coupling. *Hum Mol Gen*. 2013. 22(12):2335-49
5. Ho CY, Jaalouk DE, Vartiainen MK, **Lammerding J**. (2013). Lamin A/C and emerin regulate MKL1/SRF activity by modulating actin dynamics. *Nature*. 497(7450):507-11

Conferences/Seminars/Meetings

Work performed as part of this project has been presented at the following meetings/seminars:

- Breast Cancer Research Retreat of the Weill Cornell Medical College in New York, NY, in October 2012
- “Exploring nuclear deformability as a rate-limiting factor in cancer cell migration”, selected for a platform presentation at the 2012 Annual Meeting of the Biomedical Engineering Society (BMES) in Atlanta, GA. October 2012
- “Significance of the mechanical properties of the cell nucleus in cell migration and transit through narrow constrictions”, invited speaker. Physics of Cancer 2012 Symposium at the University of Leipzig, Germany. November 2012
- “Nuclear lamins govern nuclear deformability and modulate the ability of cells to transit through narrow constrictions during migration and perfusion”, invited keynote talk. ASME 2nd Global Congress on NanoEngineering for Medicine & Biology; Boston, MA. February 2013.
- “Nuclear lamins – their role in nuclear mechanics and mechanotransduction in physiology and disease”, invited seminar. University of Pennsylvania – Institute for Medicine and Engineering (IME); Philadelphia, PA. April 2013.
- “Nuclear Lamins – their contribution to cellular mechanics and human disease”, invited seminar. Cellular & Molecular Medicine Seminar Series at the Cleveland Clinic; Cincinnati, OH. May 2013
- “Nuclear mechanics and mechanotransduction in health and disease”. PhD Symposium of the International PhD Program Molecular Mechanisms of Cell Signaling “Cells don’t play dice”; University of Vienna and Medical University of Vienna; Vienna, Austria. June 2013.

In addition, two abstracts detailing cancer related work from our laboratory have been selected for platform presentations at the 2013 BMES Annual Meeting in Seattle, WA, in September 2013. Details will be included in the next progress report.

Cell lines

We created the following cell lines:

- MDA-MB-231 cells overexpressing either lamin A, lamin B1, lamin B2, lamin C or lamin B receptor (LBR), including unsorted cells and cells sorted for medium or high levels of overexpression

- MDA-MB-231 with reduced expression of either lamins A/C, lamin B1, lamin B2, or LBR, including heterogeneous and clonal populations
- MCF10A cells overexpressing either lamin A, lamin B1, lamin B2, lamin C or LBR
- MCF10A with reduced expression of either lamins A/C, lamin B1, lamin B2, or LBR

Microfluidic migration devices
We designed and generated novel microfluidic devices that enable visualizing cancer cells migrating or being perfused through precisely defined constrictions at high spatial and temporal resolution. We have already disseminated some of these devices to collaborating laboratories at the Cleveland Clinic and will continue to make them available to interested groups.

CONCLUSION

We have generated a panel of cells lines with systematic modulation in the expression levels of lamins and LBR, which enables us to rigorously study the effect of disturbed expression of nuclear envelope proteins. This is particularly relevant to (breast) cancer, as an increasing number of studies report altered levels of nuclear envelope proteins in a variety of cancers.[15] As part of this project, we have developed novel microfluidic devices to study the biomechanical aspects of altered nuclear envelope protein expression on the ability of cells to pass through narrow constrictions, mimicking conditions encountered during perfusion through the capillary system or migration through the interstitial space. Preliminary results from our studies indicate that reduced expression of lamins A/C, which renders the nucleus more deformable, enhances the ability of cells to transit through narrow constrictions, but may also make cells more prone to nuclear rupture in the process. At the same time, we have identified an important signaling pathway (MKL1/SRF) that is disturbed in cells lacking lamins A/C.

The research, once completed, could have important clinical implications. Analysis of expression levels of nuclear envelope proteins could be used in the diagnosis and particularly the prognosis of breast cancers, where a high fraction of cells with softer nuclei could indicate higher risk to the patient. On the other hand, increased levels of lamin A/C, as observed in one patient with an extremely aggressive form of triple negative breast cancer, might affect cancer progression by modulating other cellular functions, for example, through disturbed MKL1 signaling. While additional research is necessary to further investigate these correlations, eventually such prognostic approaches would be particularly powerful when applied to the analysis of circulating tumor cells, as it may help identify particularly aggressive subpopulations of tumor cells.

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APPENDICES

None

SUPPORTING DATA

None – all figures are included in the text above